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13. ABSTRACT (Maximum 200 words)

Most cancer treatments have severe side effects and do not protect against recurrences of the same tumor. We propose to establish an approach by which tumor cells are eradicated through selective induction of CD8⁺ T cells. Our model system will be the tyrosine kinase *HER2/neu* that is overexpressed in 30% of breast and ovarian tumors. A peptide derived from *HER2/neu* (HN654-662) has been shown to bind to HLA-A2.1 and stimulate cytotoxic T lymphocytes (CTL) that lyse primary tumors from ovarian or breast cancer. Therapies have been proposed that utilize this peptide, but the peptide has poor immunogenicity when compared to viral peptides. We have data demonstrating HN654-662 is an extremely poor HLA-A2.1 binding peptide. In a novel approach, we will make use of biophysical techniques that have recently improved sufficiently to use for experimentation. To make HN654-662 an effective therapeutic agent, we propose to first assess how the peptide binds by x-ray crystallography. The crystallographic structure will be used to design altered HN654-662 that increase binding affinity. We propose that some peptides that show increased affinity for HLA-A2.1 will also show increased affinity or avidity for a TCR specific for HLA-A2.1/HN654-662 and that these are the best candidates for therapies.

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Stimulating CTL toward *HER2/neu* Overexpressing Breast Cancer

Introduction

Cytotoxic T lymphocyte (CTL) responses to tumors in both man and animal models have been demonstrated [1-4]. Tumor cells are autologous and should be immunologically ignored, but many are recognized. These tumor cells may express mutated proteins, new proteins encoded by transforming viruses, or proteins normally expressed at low levels, but now are expressed at high levels. The primary discriminator of T cell activation is the interaction of the T cell receptor (TcR) and the MHC molecule. It is this interaction that the goals of this proposal seek to enhance.

Class I MHC molecules are ternary complexes found on the plasma membrane of nearly all cells in the body. These molecules contain a polymorphic heavy chain, β_2 -microglobulin (β_2m) and a small peptide (typically 8-10 amino acids). The heavy chains are synthesized, co-translationally translocated into the endoplasmic reticulum (ER), associate with the molecular chaperones calnexin and calreticulin and with peptide-loading molecules such as p48 and TAP [5]. Only complexes that are completely assembled with peptide and β_2m are allowed to egress from the ER into the Golgi [6]. The availability of peptide appears to be the rate limiting step in cell surface expression of class I proteins [7].

An analysis of peptides that bind to specific class I molecules show some positions in the peptide that are relatively invariant [8, 9]. These amino acids interact with substructures of the MHC molecule called pockets [10]. Originally the anchor side chains were believed to provide the majority of the free energy of binding. Subsequent analyses by our laboratories and others have clearly demonstrated that a peptide's binding ability depends on positive and negative effects from all residues within the peptide [11-13]. Possession of amino acid side chains, which would be favorable anchors, is not sufficient to make a peptide bind; nor is the absence of the residues sufficient to render a peptide unbindable. Indeed, many peptides, which seem as likely to fit, do not function as epitopes to T cells *in vivo* [14] suggesting that they do not bind to the class I molecules.

The issue of peptides binding to class I molecules is important since peptides which are not bound to class I and are not present on the surface, are not immunogenic. However, the exact effect of the affinity of peptide for class I, the stability of the complex on the cell surface and its subsequent immunogenicity is a matter of conjecture at the present time.

This interaction between heavy chain and peptide is crucial not only in the generation of CTL responses, but also in thymic selection. Class I MHC/peptide complexes are required for the egress of mature T cells from the thymus and are important in both positive and negative selection during T cell development [15, 16]. Recent experiments have shown that during thymic education the fate of the T cell (deletion or proliferation) is dependent on the affinity of the class I MHC/peptide complex and the T cell receptor [17]. Therefore, the affinity between TCR and class I MHC/peptide complex is critical for function.

A *HER2/neu* derived peptide has been identified that is recognized by autologous CTL [18]. This peptide HN654-662 (IISAVVGIL), has the HLA-A2.1 binding motif [8] and has been shown to stimulate CTL from tumor infiltrating lymphocytes derived from breast and ovarian tumors [19]. However, CTLs stimulated by HN654-662 exhibit poor cytotoxicity possibly due to the peptides poor solubility and poor binding affinity. To gain further insight into the factors that govern CTL activity, we examined the binding of HN654-662 to recombinant HLA-A2.1. As seen in our preliminary results, this peptide is extremely unusual for a peptide that stimulates CTL activity. HN654-662 marginally binds HLA-A2.1 and modifications shown to increase the affinity of other peptides ([20] and unpublished data) have little effect. Therefore, this peptide gives us the rare opportunity to use structural biology as a tool to solve an important biological problem in a timely fashion. The crystallographic structure of HN654-662 will provide information to explain the poor binding of the peptide.

Structural biology gives insights into function/importance that are not apparent from other data. For example, groups have identified peptides that do not appear to bind to class I MHC molecules in the usual manner [21]. It was the crystal structure of a HLA/peptide complex which demonstrated that the peptide extended out of the carboxyl terminus [22]. Recent advances in technology have increased the speed at which structures may be determined to the extent that structural biology is now a useful tool to probe function. For example, a class I histocompatibility complex structure can go from an idea to a finished structure solved by

molecular replacement in 2-3 months. We believe our extensive experience in the biophysical studies of class I MHC/peptide interactions and class I MHC mediated CTL killing will allow us to enter a new field, cancer immunotherapy, and make significant contributions.

Experimental Methods (Derived From Statement of Work)

Specific Aim 1. Develop HN654-662 variant peptides with improved affinity for HLA-A2.1

A The first task is to determine the co-crystal structure of HLA-A2.1 complexed with wild-type HN654-662 peptide. Objective: Complete by 12/96.

Methods: A soluble recombinant form of HLA-A2 is folded in vitro in the presence of β 2m and HN654-662. The protein is purified by gel filtration chromatography. It is concentrated to 10 mg/ml and buffer exchanged to 25 mM MES pH6.5 for crystallization trials. Initial crystals that form are crushed to make seed crystals for additional trails. Large single crystals are transferred to cryoprotectant and rapidly cool to -180°C by plunging into liquid propane. The crystals are stored as solid propane popsicles in liquid nitrogen until use. Crystallographic data are collected and the structure determined by molecular replacement methods.

Progress: We initially had great difficulty in producing large quantities of properly folded materials. This we eventually attributed to poor peptide purification. With clean peptide, we were able to obtain large crystals. Data were collected at the National Synchrotron Light Source in June of 1998 and the structure is now refined to 2.5\AA . Actual completion date, 9/98. The peptide appears to bind well at the termini, but the center is not well defined (Figure 1). We interpret this to mean that the center of the peptide assumes more than one conformation and that these conformations are not populated at one conformation much more than others. The result is an absence of interpretable electron density within the center of the peptide.

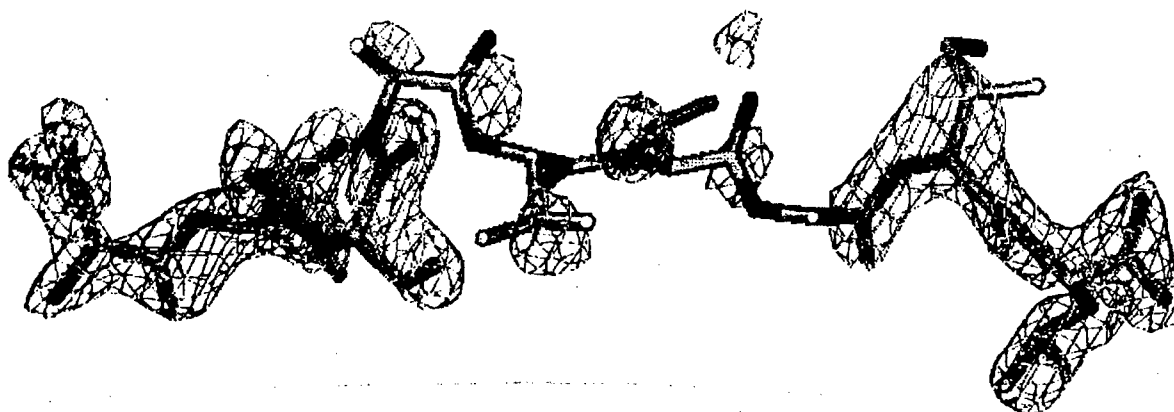


Figure 1. Electron density of peptide portion of HN654-662/HLA-A2 co-crystals. The electron density is generated from a density modified map [29]. The phases used to generate the initial map did not contain the coordinates for the peptide. Thus, the map represents unbiased electron density. The center of the peptide does not have electron density that allows us to position those amino acids within the peptide-binding cleft.

B Using the crystal structure, identify an amino acid that points down into the peptide binding cleft. Synthesize a peptide library with 20 different peptides. Fold HLA-A2 with library and isolate stabilizing peptides. Identify residue that points up towards TCR. Complete by 2/97

Methods: The library may be synthesized using standard Fmoc chemistry on a solid phase synthesizer. The position to be randomized is coupled with a mixture of 19 amino acids (cysteine left out to reduce difficulties in the folding reaction). The difficulty with the library is that salts, etc that cannot be effectively purified away inhibit folding of our protein. However, the library may be added to the folding cocktail of A2 and protein isolated as described. Folded protein will contain those peptides that allow for productive complexes. The peptides may be isolated after treatment by spinning through a centricon-3 filter apparatus (Amicon) and identified by mass spectrometry.

Progress: In the absence of the crystal structure, we have produced one library randomizing position 3. Position 3 has been shown to be a secondary anchor in many peptides. As the peptide only has one polar residue and it is the serine at P3, we decided to substitute the first position isoleucine with lysine to improve solubility of the peptide library. The library was used in our in vitro folding reaction and large quantities of A2 were isolated. The protein is not very stable however. Warming the mixture to room temperature resulted in complete denaturation of the complex. We are gearing up to repeat this experiment and will keep it at 4 °C until we are ready to isolate peptides.

With the crystal structure in hand, we have identified one residue that points mostly sideways (Figure 2). The remaining residues within the cleft are not well defined (as described above and in the conclusions) and we are unable to predict which residues should be pointing down and which up. Therefore, we are making simple substitutions to map out the TcR contacts. The residues that do not appear to be important for TcR recognition will be our next targets for library formation.

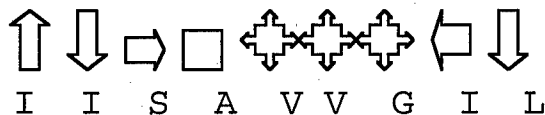


Figure 2. Orientations of the amino acids of H654-662 when bound to HLA-A2. Arrows pointing in all four directions: we cannot reliably position these amino acids; Up arrow: towards TcR; Down arrow: towards beta pleated sheet; Right arrow: towards $\alpha 2$ α helix; Left arrow: towards $\alpha 1$ α helix.

*C Chemically synthesize peptides that improve stability and determine thermostability.
Complete by 4/97.*

In the absence of the crystal structure, we made anchor substitutions that have been shown to increase affinity of peptides to class I MHC in the past. With the crystal structure in hand, we have found an amino acid that points under the $\alpha 2$ α helix that could accommodate a larger side chain. We synthesized all of these peptides and tested for increased binding affinity by three methods. The first method is circular dichroism spectroscopy. The change in the circular dichroic signal as a function of temperature is followed using soluble recombinant protein. The temperature at which 50% of the protein is denatured is the T_m . The T_m has been shown to be proportional to the affinity of the peptide [30]. The second method is to measure a relative binding constant, by adding peptide exogenously to T2 cells that lack a functional peptide transporter. These T2 cells take up a large fraction of these peptides. HLA-A2 on the surface of these cells take up the peptide and by virtue of the binding, also stabilize the HLA-A2. The A2 on the surface can be followed with antibodies and the quantified by flow cytometry. The third method is to measure the half-lives of the A2 on the surface of the cells using the T2 cells described above. The cells are incubated with peptide. Brefeldin A is added to halt vesicular transport. Therefore, no new A2 can come to the surface and the A2 on the surface remains there until the protein denatures (presumably as the peptide falls out of the complex).

The amount of class I on the surface is followed over time to determine the physiological lifetime of the complexes on the cell surface.

Progress: We have synthesized a number of different peptide variants and compared them with other described physiological epitopes from HER2/neu. Figure 3 shows typical melting curves for some of these complexes. The results are summarized in Table 1.

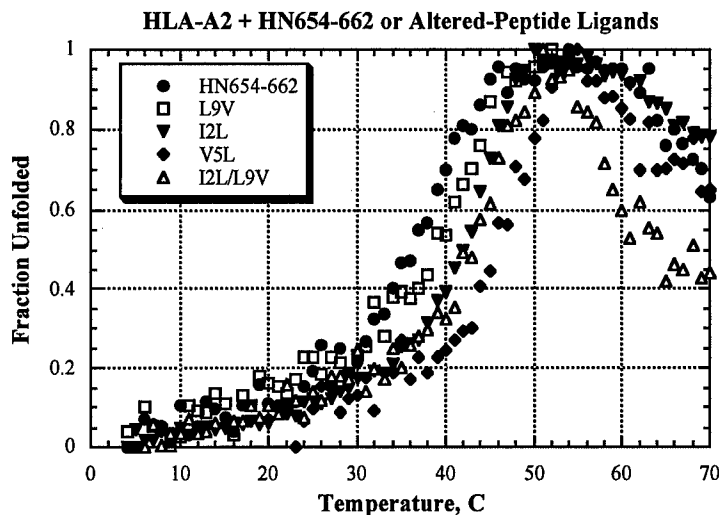


Figure 3. Melting curves for Hn654-662 and APL bound to HLA-A2. The change in circular dichroic signal at 218 nm was measured as a function of temperature in an AVIV 62DS spectropolarimeter. The concentration of protein was between 4 and 12 μ M. Each curve is the average of at least three measurements from two or more individual protein preparations. The point at which 50% of the protein is denatured is the T_m . The error associated with the T_m is approximately one degree.

<u>HLA-A2 Restricted Peptide</u>		<u>T_m (°C)</u>
IISAVVGIL	(HN654-662)	36.4
ILSAVVGIL	(I2L)	42.2
IISAVVGIV	(L9V)	38.8
ILSAVVGIV	(I2L/L9V)	42.5
IISALVGIL	(V5L)	45.8
ILSALVGIV	(I2L/V5L/L9V)	38.5
SIISAVVGI	(S1)	44.2
IISAVVGILL	(L10)	41.3

Table 1. Melting temperatures for HLA-A2 bound to HN654-662, Altered-Peptide Ligands and some other identified *Her2/neu*-derived immunogenic peptides as measured by circular dichroism.

We have also measured binding using the T2 cell surface assembly assay to be sure that there were no artifacts that were a result of the assay being used. There were no qualitative differences.

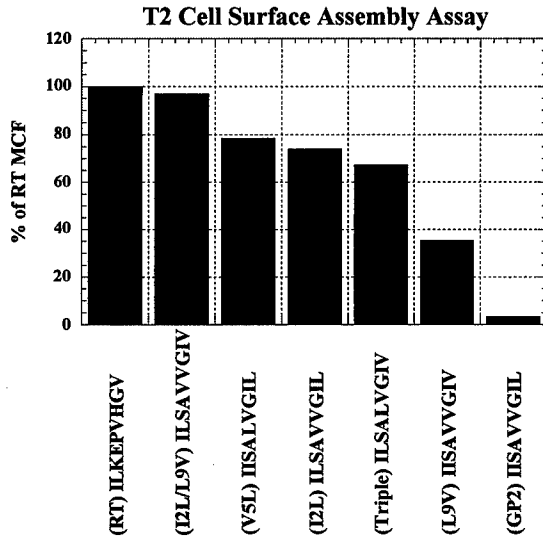


Figure 4. Relative binding of the altered-peptide ligands as measured by flow cytometry. Briefly, T2 cells are incubated with peptide (50 μ M in this case) overnight in serum free media overnight. In the morning, excess peptide is washed away and the cells are stained with an anti-class I MHC monoclonal antibody (BB7.2). After washing, the cells are incubated with FITC-conjugated anti-mouse antibody. The level of cell surface expression is examined by flow cytometry. The mean channel fluorescence is calculated for each peptide-pulsed sample and normalized to the mean channel fluorescence for RT ILKEPVHGV (our positive control).

We believe that the most relevant in vitro assay that reflects biological importance is the time the peptide/complex remains on the surface of the cell for CTL to "see" it. We examined the biological half-lives using modification of the T2 cell surface assembly assay. A representative experiment is shown in Figure 5. As can be seen, the complexes do not remain on the surface for great periods of time. HN654-662 has a half-life of approximately 20 minutes. RT for comparison has a half-life of 10 hours. We are attempting to generate HN654-662 variants with half-lives approaching that of RT. We are measuring half-lives of our new variants now.

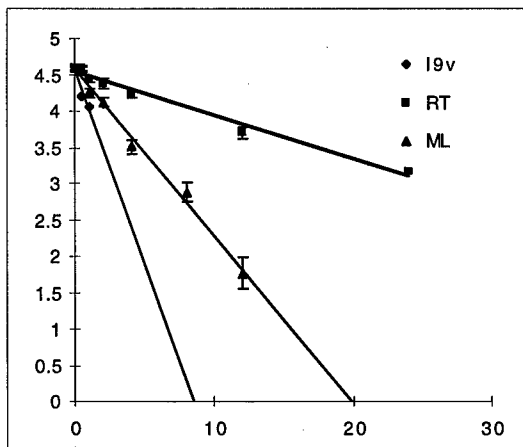


Figure 5. Representative half-lives experiments for cells incubated with RT (ILKEPVHGV), ML (MLLSVPLL) or the L9V variant of HN654-662 (IISAVVGIV). Briefly, cells are treated as described above except in the morning, the cells are washed with Brefeldin A (BFA) to halt vesicular traffic. Aliquots of cells are removed over time and stained as described above to determine the level of class I MHC remaining on the cell surface

D. Chemically synthesize cysteine mutant sequence and test for binding to HLA-A2 in in vitro assay. Link to Biacore chip and measure on and off-rates to HLA-A2.1. Complete by 9/97.

Methods: We will synthesize the peptides through standard FMOC chemistry and will test them for binding as described above.

Progress: We have not begun this aim. We intend to start this as soon as we have isolated T cell clones as described below.

Specific Aim 2. Screen improved epitopes for enhanced affinity for the T cell receptor.

A. Isolate murine CTL lines specific for HN654-662. Line by 12/96 clone by 6/97. Approx. 75 mice.

Methods: CTL are most readily made by stimulation by professional antigen-presenting cells. We will isolate dendritic cells from PBLs of A2/K^b mice and stimulate T cells with Hn654-662 and altered-peptide ligands. Lines will be generated by repeated in vitro stimulations, clones by limiting dilution or FACS.

Progress: Due to difficulties with agreements on the use of the HLA-A2/K^b transgenic mice that we obtained from the Scripps Institute, we could not work on this aim until similar mice were either made or obtained elsewhere. Dr. Roland Tisch (UNC Microbiology and Immunology) has made mice transgenic for this molecule, but it is on the FEB background (q haplotype). We are presently evaluating how to proceed from here.

In the absence of mice that were suitable for this work, we used human PBLs. HLA-A2 donors were obtained from a different project. Dendritic cells from these donors were used to stimulate T cells using Hn654-662. T cells were obtained that recognized HN654-662 specifically and recognized some altered-peptide ligands better. Figure 6 shows the first such experiment. We are stimulating with altered-peptide ligands now and will test for reactivity against HN654-662 next.

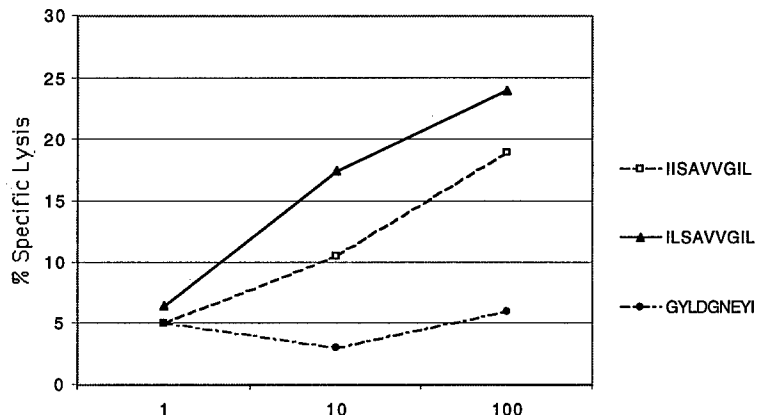


Figure 6. CTL recognize I2L better than wild-type HN654-662. Naïve T cells stimulated with HN654-662 bound to autologous dendritic cells cultured in vitro are tested in the standard 4 hr chromium release assay.

2L is Recognized More Efficiently Than HN654-662

B. Transfect LINE1 with HER2/neu and HLA-A2.1 and select. Complete by 12/96

Methods: Using cDNAs for Her2/neu and HLA-A2/K^b, make stable transformants of LINE1 for inoculation into the Tg A2/K^b mice.

Progress: We have constructs for *HER2/neu* and HLA-A2.1, but because we cannot use the mice that we had originally planned to use, we have not transfected LINE1 with those genes. We are presently using human T cells stimulated with wild-type HN654-662 or APL. We will use these CTL to test against tumor cell lines for reactivity. If a reasonable animal model may be constructed, we will use it. In the mean time, we intend to make human T cell clones to isolate TcR for the following experiments.

C. Test CTL reactivity of variant peptide determined from 1 using CTL clone. Complete by 12/97

Methods: Using RMA-S cells or syngeneic B cells, peptide pulse and perform the standard 4 hour chromium release assay on APL.

Progress: As described above, we have switched our efforts to isolating a human T cell clone and we are testing CTL reactivity against HLA-A2-transfected C1R with HN654-662 and APL.

We have successfully shown that CTL raised towards HN654-662 from a naïve individual are capable of recognizing HLA-A2-transfected C1R and recognize some APL better (Figure NEXT). We are now stimulating with APL and testing for reactivity to wild-type HN654-662.

D. Test protection of mice with variant peptide immunization. First trial five concentrations/ 5 mice per concentration. Repeat with vaccinia construct if required. Repeat changing order of treatment (add tumor then immunize). Complete by 6/98.

Methods: We cannot use the mice of choice, the mice that are transgenic for HLA-A2/K^b are presently on the wrong genetic background. We have altered our plans to use human T cells and test reactivity against established tumor cell lines and primary tumors.

Progress: We have procured some of the tumors and have generated CTL lines specific for HN654-662. We will test reactivity using the standard chromium release assay soon.

E. Produce soluble TCR. Complete by 6/98.

Methods: Isolate cDNA using Fast-Track kit (Invitrogen). Clone and determine sequence. Using PCR make constructs for expression in the baculovirus system and also as fusion proteins of variable domains using the TrcThioHis system (Invitrogen). Isolate by metal chelation chromatography and other chromatographic methods as required. Test for proper folding using monoclonal antibodies that recognize the corresponding alpha and beta chains.

Progress: We have expressed other T cell clones using this process successfully and are now prepared to do this when a T cell clone is obtained.

F. Test affinities of class I/ peptide complexes with soluble TCR. Complete by 12/98.

Methods: Surface plasmon resonance will be used to measure on and off rates of complexes fixed to the surface of appropriate chips. In each case, we will engineer E. coli BirA recognition sites to specifically biotinylate the carboxyl terminus of each protein. Then each protein individually will be bound to strepavidin-coated chips. On and off rates should be independent of which protein is coupled to the chip.

Progress: We have successfully engineered BirA recognition sequences to the carboxyl end of HLA-A2.1. These proteins fold in vitro as well as the wild-type A2 sequences. We will perform the same to the T cell clone isolated.

G. Test class I peptides identified in 1 as vaccines with transgenic mice. Complete by end of grant.

Methods: Peptide-pulsed dendritic cells (DCs) will be used as vaccines to generate specific T cell responses. The DCs may be used to test protection in a tumor challenge model or tested for their ability to slow or reduce the growth of a tumor.

Progress: We can now successfully culture DCs from humans and mice. We have not decided how to approach this aim due to a good animal model. We are collaborating with Dr. Jon Serody to test one of the APL as a phase 1 clinical trial for breast cancer. This test will prove to be significantly more informative as our ultimate aim is to provide a relative human immunotherapeutic.

Conclusions

We have made significant progress within the last year. We have determined the crystallographic structure of one of the worst binding ligands for HLA-A2 measured to date. We have used that information to design peptides that have improved binding to class I MHC. We are testing their biological relevance now. We have cultured human dendritic cells that stimulate human CTL against the wild-type HN654-662 from a naïve donor. We have shown that those CTL recognize variant peptides better than the wild-type sequence. We have redesigned the experimental scheme to examine T cell recognition of tumor cell lines and primary tumors. After we finish the evaluating the CTL reactivity, we will be better able to gauge the significance of these data. It is significant as a crystallographer to use data and design new ligands. It is rare when we actually succeed in designing ligands that bind as we expect them to. However, it is not biologically exciting unless we can stimulate significantly more CTL that recognize the wild-type ligand with the altered peptide ligands. We expect the next year to be even more fruitful than this past year in terms of the potential yield.

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Appendices

None



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

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PHYLLIS M. RINEHART
Deputy Chief of Staff for
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